Transgenic Plants: A Production System for Industrial and Pharmaceutical Proteins

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2 The Production of Recombinant Glycoproteins with Defined Non-Immunogenic Glycans

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INTRODUCTION

Higher organisms synthesize glycoproteins with *N*-linked glycans (oligosaccharides) attached to certain Asn-X-Ser (or Thr) tripeptides and *O*-linked glycans attached to Ser, Thr or Hyp residues; these glycoproteins are associated with the membranes of the secretory system, accumulate within specific compartments (ER, vacuoles, lysosomes, endosomes) or are secreted from the cells. The glycans attached to these glycoproteins contain a variety of sugar residues linked in linear or branched structures that can assume many different configurations. Although the glycans of plants and mammals have certain common features, they also differ in very fundamental ways. The functions of glycans on glycoproteins have been studied quite extensively and it is now clear that they do not have a unique function, but rather impart to the protein to which they are attached new properties related in some way to the function of that protein. The functions of glycans can be divided in intermolecular, e.g. recognition of other molecules, or intramolecular, e.g. protein folding or solubility, categories (Fig. 2.1; reviewed in Stanley 1984). Although in some cases polypeptides without their normally attached glycans have the same biological activity, e.g. catalytic activity, for the vast majority of glycoproteins the glycans are essential to their effective functioning in the organism. Much less is known about glycoproteins of plants than of mammals, but a complete review of plant glycan structure, biosynthesis and function has recently been published (Sturm 1995). Therefore, only the essential features of *N*-linked glycan structure and biosynthesis will be discussed here.

Many proteins, such as those found in blood plasma, are of great pharmaceutical interest and the majority of the proteins in blood plasma are glycoproteins (Fig. 2.2). Currently they are obtained by purification from blood plasma, but this method of production has several drawbacks. Not only is plasma in short supply, but it is often contaminated with viruses and

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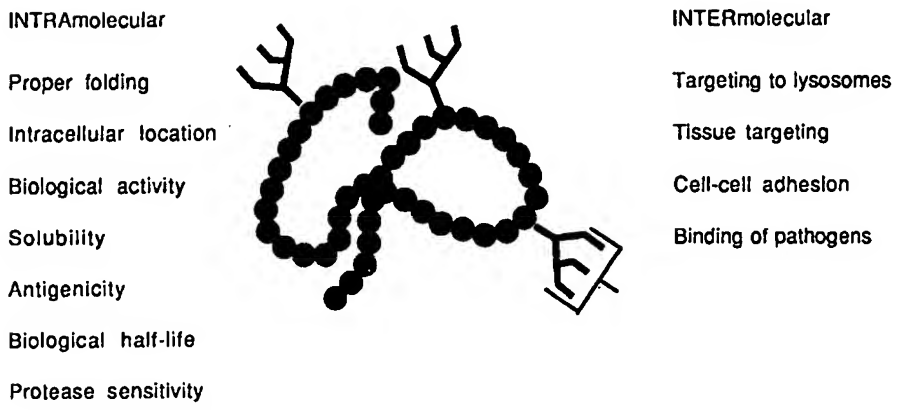


Figure 2.1 Potential roles of carbohydrates in glycoproteins

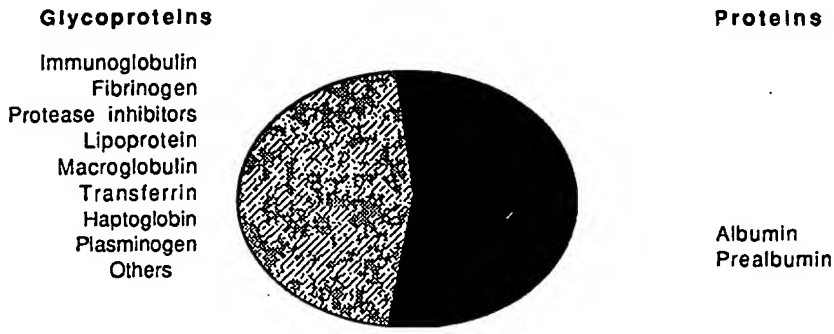


Figure 2.2 Composition of human plasma

prions, disease agents which may be difficult to separate from the proteins of interest. An attractive alternative is to produce the proteins in transgenic cells or organisms. Recombinant DNA technology has already been used successfully and a variety of these proteins are now produced in large bioreactors using mammalian cells, and are available as biopharmaceutical products. The drawbacks of this approach are the cost of production on an industrial scale, the low yield and the need for extensive purification and quality control testing.

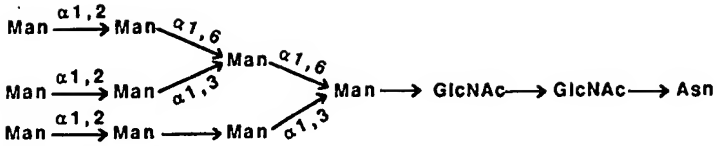
Recombinant proteins can also be produced in heterologous host cells, including bacteria, yeast, insect, plant or mammalian cells. All these cell systems correctly synthesize the polypeptide of a heterologous protein when the cDNA encoding the protein is introduced into the cells with an appropriate expression vector. However, when post-translational modifications of the recombinant polypeptide, and particularly glycosylation, are required to obtain biological activity, not all these host cells have the same capacity to produce active therapeutic glycoproteins.

What is the best system, and what are the constraints for the production of these glycoproteins? Bacteria do not glycosylate proteins, so glycoproteins cannot be produced in *Escherichia coli*. The glycosylation mechanisms in the higher organisms are evolutionarily conserved but differ in detail. Yeast, insect, mammalian and plant cells all attach high-mannose glycans to the same Asn residues, but differ in the trimming and further modification of the glycans in the Golgi. Thus no heterologous system will reproduce the mammalian glycans exactly. Because plants are well suited for the production of large amounts of glycoprotein at a low cost, they are gaining acceptance for the expression of recombinant therapeutic proteins (Moffat 1995). It is therefore necessary to examine to what extent the glycans of human glycoproteins synthesized in plants will differ and how these differences can be minimized or eliminated.

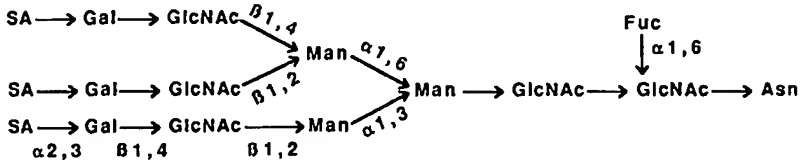
COMPLEX ASN-LINKED GLYCANS OF PLANTS AND MAMMALS ARE DIFFERENT

Specific Asn residues in Asn-X-Ser or Asn-X-Thr tripeptides in plant proteins can function as glycan acceptors just as in mammalian and yeast polypeptides. This short sequence is recognized by the ER-localized oligosaccharyl transferase that transfers $Glc_3Man_9GlcNAc_2$ groups from dolicholpyrophosphate to nascent polypeptide chains. This oligosaccharide will then mature during the intracellular transport of the glycoprotein through the secretory pathway. As in mammalian cells, the glycan processing machinery of plant cells is located in the endoplasmic reticulum (ER) and the Golgi apparatus. However, plant and mammalian glycans differ in their structure and sugar composition. The Asn-linked glycans present on plant glycoproteins can be divided into two classes: high-mannose (also called polymannose) and complex-type glycans. Both types have a common core consisting of two *N*-acetylglucosamine (GlcNAc) residues linked at the reducing end to Asn and at the non-reducing end to the β-linked mannose (Man) of the core. The high-mannose glycans have 5–9 mannose residues in the typical branching pattern shown in Figure 2.3. Most plant complex glycans have only three mannose residues and additional fucose (Fuc) and xylose (Xyl). Some, such as the large biantennary complex glycan found in the secreted protein laccase, have additional GlcNAc, fucose and galactose (Gal) residues. The residues themselves, or the manner in which they are linked, differ from the complex glycans of mammalian glycoproteins but resemble those of invertebrate glycoproteins. Thus, plant complex glycans have $\alpha 1 \rightarrow 3$ fucose on the proximal GlcNAc, as opposed to $\alpha 1 \rightarrow 6$ fucose in mammals, and $\beta 1 \rightarrow 2$ xylose on the β-linked mannose of the core. Insect and mollusc complex glycans resemble the complex glycans found on plant glycoproteins rather than those of animal glycoproteins.

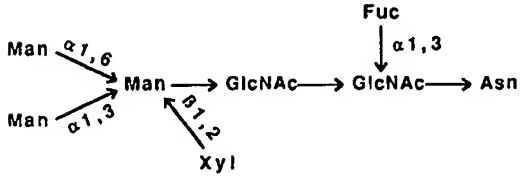
Plant glycoproteins have been found to be uncommonly antigenic in mammals, and the likely cause is these two residues/linkages, not found on



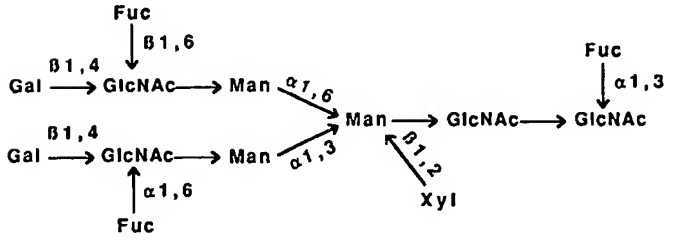
High mannose — plant and mammal



Complex — mammal



Complex — plant (common)



Complex — plant (laccase type; uncommon)

Figure 2.3 The structures of Asn-linked high-mannose and complex glycans of plants and mammals

mammalian glycoproteins. These types of glycans will be present on recombinant human glycoproteins produced in plants. As a consequence, the recombinant glycoproteins produced in plants used as pharmaceuticals may result in sensitization to these unique plant antigens. However, this difficulty is not specific to plants: none of the transgenic host cell systems available for the production of recombinant human serum glycoprotein will produce human glycoproteins with the same glycans normally found on those proteins produced in human cells.

One of the advantages of plants as production systems for recombinant human glycoproteins over systems such as insect cells or yeast, is our greater knowledge of the protein glycosylation machinery and the availability of mutant plants, allowing us to define strategies to produce recombinant proteins with more mammalian-like glycans.

BIOSYNTHESIS OF COMPLEX GLYCANS IN PLANT CELLS

The biosynthesis of Asn-linked glycans starts in the ER with the assembly of $GlcNAc_2Man_9Glc_3$ glycans on dolicholpyrophosphate. The dolichol lipid anchors the glycan to the membrane. The exact details of the biosynthesis have not been worked out for plants, but it is highly likely that these are analogous to those found in mammals. Sugars are first transferred from nucleotide sugar donors one by one to dolicholpyrophosphate on the cytoplasmic side of the ER membrane. When the glycan reaches the size $GlcNAc_2Man_5$, it flips to the other side of the membrane and additional sugars are transferred from nucleotide sugar donors via dolichol to the growing glycan group in the lumen of the ER. The completed $GlcNAc_2Man_9Glc_3$ group is transferred *en bloc* to the nascent polypeptide chain, and the three Glc residues are removed almost immediately by two different glucosidases. Glycoproteins purified from the ER always contain high-mannose glycans without Glc residues. When proteins are transported to the Golgi apparatus their glycans may be modified by the sequential action of glycosidases and glycosyltransferases to yield complex glycans.

During the last decade considerable progress has been made in the characterization of the enzymes involved in the processing of *N*-linked glycans of plant glycoproteins and their compartmentation in the cisternae of the Golgi. *In vitro* enzyme assays with defined glycopeptide substrates (Johnson and Chrispeels 1987), the use of inhibitors of specific enzymatic steps (Elbein 1991), the analysis of an *Arabidopsis thaliana* mutant that lacks a specific Golgi enzyme (von Schaewen et al. 1993) and the use of immunopurified antibodies specific for the $\beta \rightarrow 1,2$ xylose and $\alpha \rightarrow 1,3$ fucose of plant *N*-glycans (Faye et al. 1993; Fitchette-Lainé et al. 1994) led to the model for the pathway of glycan processing shown in Figure 2.4. Enzymatic assays of solubilized Golgi fractions with defined glycopeptide substrates indicated that in plants, as in animals, the first modification steps include the action of α-mannosidase I and GlcNAc-transferase I (GnT-I). The key role of GnT-I for the synthesis of

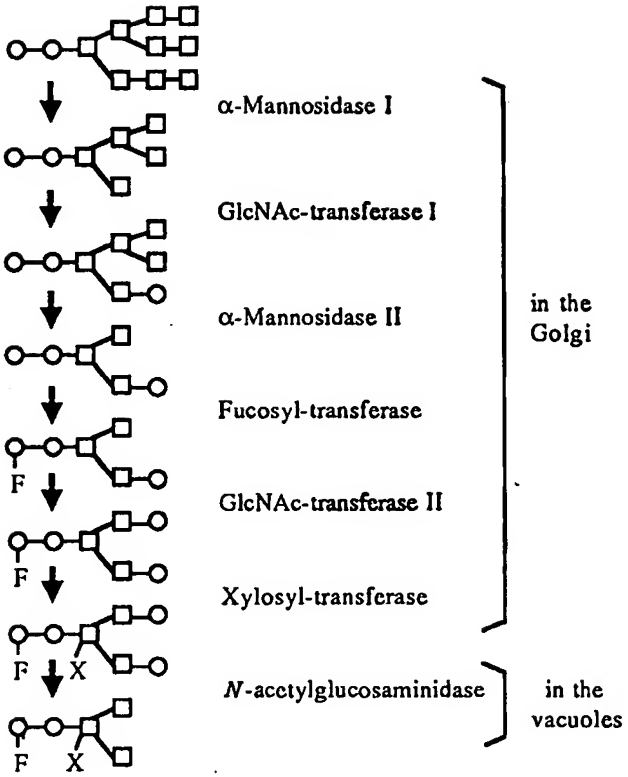


Figure 2.4 Steps in the conversion of a high-mannose glycan to a typical complex glycan

complex glycans was confirmed by the isolation of a mutant *Arabidopsis thaliana* strain which synthesizes glycoproteins that have high-mannose glycans but no complex glycans. The glycans in these mutant plants do not contain fucose or xylose (von Schaewen et al. 1993). GnT-I is the key enzyme in complex glycan biosynthesis in plant cells as well as in animal cells, and this step is common to both systems. Modifications downstream of GnT-I are responsible for the specific features of plant complex glycans. These modifications, such as $\alpha \rightarrow 1,3$ fucosylation and $\beta \rightarrow 1,2$ xylosylation, which are responsible for plant complex glycan immunogenicity, can only occur in cells where GnT-I is active.

THE COMPLEX GLYCANS OF PLANT GLYCOPROTEINS ARE UNUSUALLY IMMUNOGENIC

When plant glycoproteins are injected into animals to produce antibodies, the resulting sera are often 'non-specific' (see Kaladas et al. 1983; Lainé and Faye 1988; McManus et al. 1988; Laurière et al. 1989). This means that on immunoblots the antibodies recognize numerous proteins. Such sera usually contain antipolypeptide as well as antiglycan antibodies. Because $\beta 1 \rightarrow 2$ xylose linked to core mannose and $\alpha 1 \rightarrow 3$ fucose linked to core GlcNAc are not found on mammalian glycoproteins, these plant glycan epitopes elicit unusually strong immunological reactions when plant glycoproteins are used as antigens. The different sets of antibody molecules can usually be separated by fractionating the serum on one or more affinity columns. Faye et al. (1993) used this approach to produce 'antixylose' and 'antifucose' serum fractions. The antixylose fraction was obtained by passing the serum over a column of honey bee venom phospholipase A_2 (commercially available). The glycan on this insect protein has $\beta 1 \rightarrow 2$ xylose, but contains no fucose. The bound fraction was eluted and constituted the antixylose serum fraction. A parallel approach was used to produce antifucose antibodies.

For practical purposes it is necessary to remove glycans, especially complex glycans, before plant proteins are used as antigens. Complete removal can only be accomplished with trifluoromethyl sulphonate (TFMS) using anhydrous conditions (Edge et al. 1981). This procedure is not difficult and works reliably if care is taken to store the reagents under anhydrous conditions. The use of enzymes to remove complex glycans from plant glycoproteins is unreliable. Endoglycosidase H (Endo H) does reproducibly remove high-mannose *N*-linked glycans. However, there are no suitable enzymes to remove the complex glycans of plant glycoproteins. The presence of the $\alpha 1 \rightarrow 3$-linked fucose on the proximal GlcNAc interferes with the action of the enzymes that remove complex glycans from mammalian glycoproteins (Tretter et al. 1991; Altmann et al. 1995).

When human glycoproteins are produced in plants they will have the highly immunogenic glycans typically found on plant glycoproteins. These antigens are not novel for humans, who are exposed daily to such antigens in food and pollen. However, if people have prolonged exposure to large quantities of these antigens, as may be required by certain therapies, sensitization may result. This is exemplified by baker's asthma, an allergic asthma that is common among workers with occupational exposure to cereal flour. A family of small cereal proteins known as α-amylase inhibitors are major allergens associated with baker's asthma. The glycosylated forms of these proteins have much greater allergenicity than the unglycosylated forms (Gomez et al. 1990). Among the glycosylated forms, by far the strongest allergens are those that have the typical small complex glycans found on plant glycoproteins (Garcia-Casado et al. 1996).

From this information on plant complex glycan biosynthesis, two strategies

to prevent the formation of highly immunogenic plant complex glycans on recombinant proteins emerge. The first possibility is to retain the recombinant human glycoprotein in the ER, so that the glycans that are added in the ER are not modified in the Golgi. The second strategy, which allows for storage of the proteins downstream from the Golgi, i.e. in the vacuole or the apoplast, is to modify the enzyme complement of the Golgi by knocking out enzymes (mutagenesis) and/or adding new enzymes (transformation).

RETENTION IN THE ER

In most plant cells the vacuole is the major protein storage compartment. Although the vacuoles of many cell types contain very little protein, under specific conditions and in certain cell types abundant amounts of protein accumulate in this large compartment (Fig. 2.5). Proteins that accumulate there will usually have passed through the Golgi apparatus, and if they are glycoproteins they will have acquired complex glycans (Vitale and Chrispeels 1992). Vacuolar glycoproteins always have small complex Asn-linked glycans (see Fig. 2.3).

As illustrated for storage proteins in the endosperm of certain cereal seeds, e.g. zeins in maize, the ER can also be a large storage compartment and it could be used advantageously for recombinant glycoprotein accumulation in transgenic plants. In eukaryotic cells, proteins that reside in the lumen of the ER have typical C-terminal retention signals: KDEL or HDEL (Pelham 1990). This is also the case in plant cells (see Chrispeels 1991). When secretory proteins are modified so that they carry the HDEL or KDEL motif at their C-terminus they may be retained in the ER when expressed in a heterologous system. However, the mere presence of this retention signal is no guarantee that the protein will be retained in the ER. Successful retention was obtained with pea vicilin KDEL (Wandelt et al. 1992) and with sweet potato sporamin HDEL (Faye and Gomord, unpublished). However, bean phytohaemagglutinin KDEL (Herman et al. 1990) and bean phaseolin HDEL and KDEL (Pueyo et al. 1995) were transported to the vacuole in spite of the presence of the ER retention signal (Table 2.1). These results suggest that the properties of the proteins and of the heterologous cells in which the genes are being expressed may also play a role in whether or not retention occurs. When there is a large amount of accumulation, as in the case of vicilin KDEL in leaves of alfalfa

Table 2.1 Subcellular fate of vacuolar proteins with KDEL/HDEL extensions

Vicilin – KDEL	Retained in ER
Sporamin – HDEL	Retained in ER
Phytohaemagglutinin – KDEL	Proceeds to the vacuole
Phaseolin – *c-myc* – HDEL	Proceeds to the vacuole

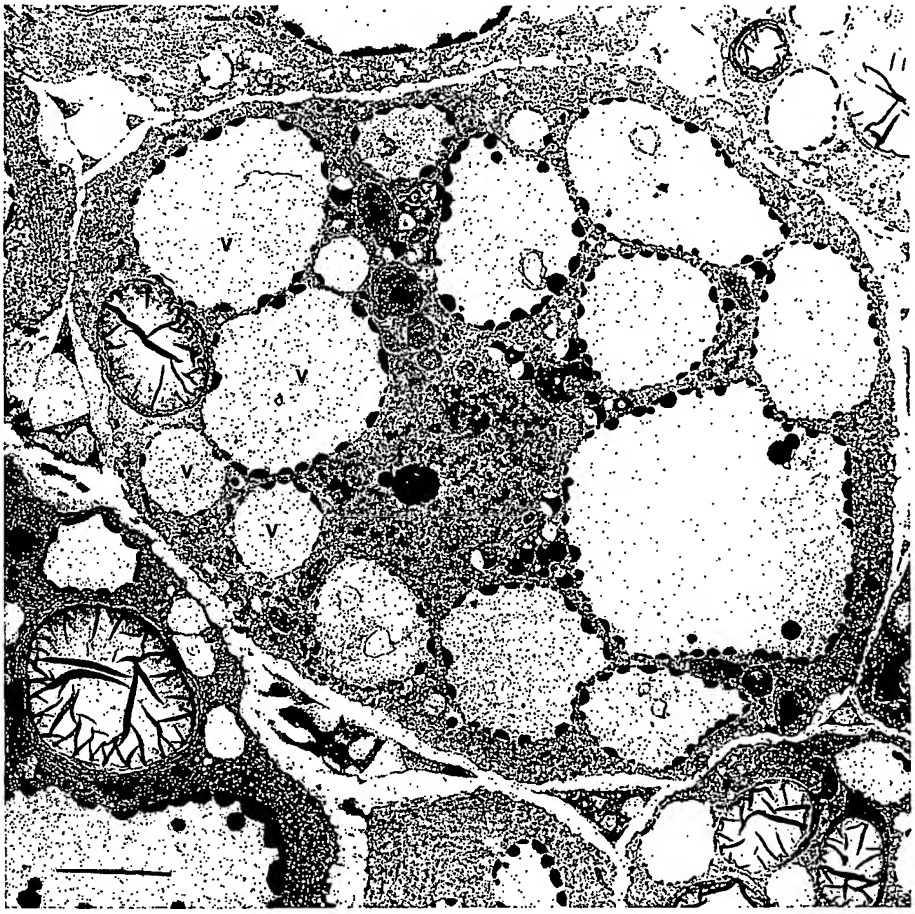


Figure 2.5 The vacuoles (V) of parenchyma cells in developing pea cotyledons form a large compartment for the storage of recombinant proteins. Electron-dense material in the vacuoles is pea seed storage protein. Bar = 5 μm. (Courtesy of Stuart Craig, CSIRO, Canberra, Australia)

(Wandelt et al. 1992) recombinant protein accumulates in ER-derived 'protein bodies' in the cytoplasm. In other cases no KDEL is necessary to cause the accumulation of protein in a greatly expanded ER. For example, Bagga et al. (1995) demonstrated that 15 kDa zein accumulates in unusual protein bodies in the cytoplasm of tobacco leaves (Fig. 2.6). These protein bodies are most likely ER derived and are separated from the cytoplasm by an ER membrane. It remains to be demonstrated whether the glycans on proteins that accumulate in this distended ER compartment have indeed not been modified by Golgi enzymes. Why proteins such as zeins, which do not have recognizable ER-retention motifs, accumulate in the ER also still needs to be elucidated (Galili et al. 1993).

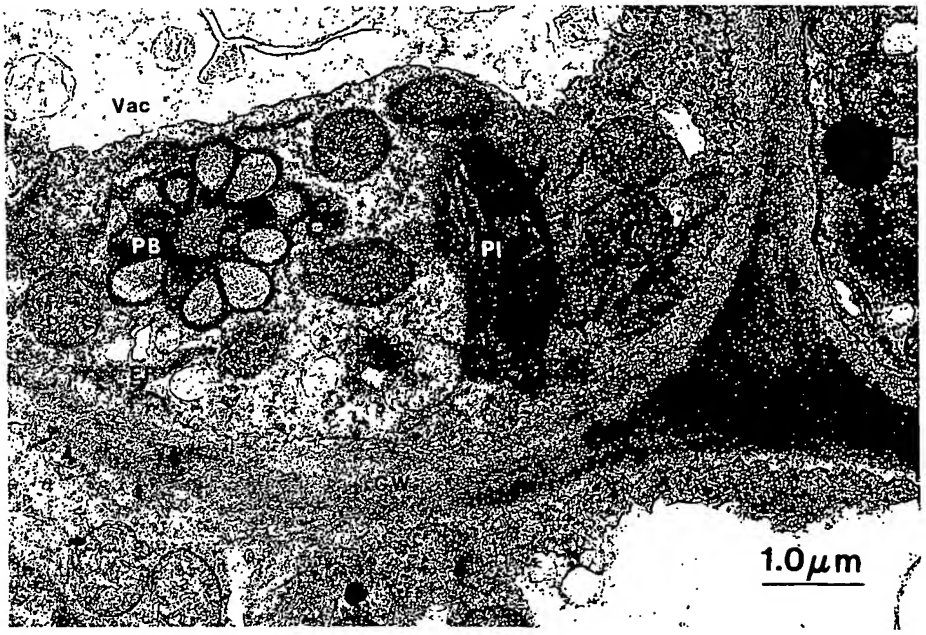


Figure 2.6 Tobacco mesophyll cell showing a multilobed ER-derived protein body (PB) filled with recombinant zein (a maize endosperm storage protein). ER = endoplasmic reticulum; Vac = vacuole; cw = cell wall; PI = plasmid; Mi = mitochondrion. (Courtesy of Suman Bagga and Champa Sengupta-Gopalan, New Mexico State University)

KNOCKING OUT GENES THAT ENCODE GOLGI ENZYMES

The second strategy to produce glycoproteins with non-immunogenic glycans is to use plants that lack one or more Golgi enzymes involved in Golgi modification of *N*-linked glycans. Several glycosylation mutants of mammalian cells, which have a defect in a particular step of protein glycosylation, have been isolated and characterized (see Stanley 1984 for review). These mutants are defective in biosynthesis of the mature oligosaccharide attached to dolicholpyrophosphate or in glycan processing, or show an altered terminal glycosylation pattern. In most cases the functional consequences of glycosylation mutations were difficult to assess, which may be due to the fact that the mutants are cultured cells and not intact organisms. Some mutant cells defective in *N*-glycan biosynthesis show a conditional-lethal phenotype, whereas others have defects in intracellular trafficking (Stanley 1984). In contrast, Chinese hamster ovary (CHO) cell mutants that lack GnT-I activity, and as a consequence have no complex carbohydrates, are quite healthy and synthesize many glycoproteins that are biologically active, despite their truncated carbohydrates (Kumar et al. 1990).

Whole-mammal glycosylation mutants are very rare and were discovered

as human genetic disorders such as HEMPAS, a deficiency in α-mannosidase II and/or low levels of GlcNAc-transferase II (Fukuda 1990; Fukuda et al. 1990). In these rare cases, mutations in protein glycosylation seem to have severe effects on the viability of the mutants. The feasibility of obtaining mature plants with altered glycans was demonstrated by von Schaewen et al. (1993), who isolated a mutant *A. thaliana* strain that lacks GnT-I, the first committed step in the modification pathway. *Arabidopsis* seeds were mutagenized with a chemical mutagen, planted and the resulting plants selfed. The seeds obtained in this way were planted and extracts of a single leaf of 8000 plants were screened with an antiserum that recognizes complex glycans. Four mutant plants were obtained that showed no staining with the serum.

The glycoproteins in callus tissues derived from two of these mutants bound to concanavalin A, indicating the presence of high-mannose glycans, but failed to incorporate any radioactive fucose. The glycans could be released by treatment with Endo H and the majority have a $Man_5GlcNAc_2$ structure. In the presence of deoxymannojirimycin, an inhibitor of α-mannosidase I, the mutant cells synthesize $Man_9GlcNAc_2$ and $Man_8GlcNAc_2$ glycans, suggesting that the biochemical lesion in the mutant is not the biosynthesis of high-mannose glycans in the endoplasmic reticulum, but their modification in the Golgi. Direct enzyme assays of cell extracts show that the mutant cells lack GnT-I activity, the first enzyme in the pathway of complex glycan biosynthesis (see Fig. 2.4). The mutant plants are able to complete their development normally under several environmental conditions, suggesting that complex glycans are not essential for normal developmental processes. This is in contrast to the mouse, where a knockout of a gene encoding the same enzyme is lethal at an early stage in embryogenesis (Ioffe and Stanley 1994; Metzler et al. 1994). The finding that the plants have a normal phenotype indicates that complex glycans in plants must play fundamentally different roles than in mammals. The practical consequence of this discovery is that it should be possible to knock out any enzyme in this pathway and produce glycans without either fucose or xylose. By crossing the complex glycan-deficient strain of *A. thaliana* with a transgenic strain that expresses the plant glycoprotein phytohaemagglutinin, von Schaewen et al. obtained a unique strain that synthesized phytohaemagglutinin with two high-mannose glycans, instead of one high-mannose and one complex glycan. This shows that proteins encoded by transgenes can be synthesized with customized glycans. After knocking out genes encoding GnT-I in cereals or legumes it would be possible to store recombinant human glycoproteins in compartments downstream from the Golgi, i.e. the apoplast or the protein bodies, without risk of complex glycan modifications on these glycoproteins.

ADDING NEW GENES

Another difficulty found in a variety of heterologous host cells, and particularly plant cells, is that they do not synthesize sialic acid containing

glycoproteins. Sialylation of blood serum glycoproteins has been shown to increase their half-life (Gross et al. 1987) in the circulation and it may therefore be desirable to obtain plant cells able to produce sialylated glycoproteins. Most mammalian glycosyltransferases, and particularly $\alpha \rightarrow 2,6$ sialyltransferase, have been cloned and the feasibility of a strategy to enlarge the spectrum of glycosyltransferases in plant cells has been recently demonstrated. To find out if a mammalian Golgi enzyme could complement the *A. thaliana* mutant described above, Gomez and Chrispeels (1994) transformed mutant plant cells that lack the plant GnT-I with the cDNA for human GnT-I. Plant cells expressing this human cDNA were able to convert high-mannose glycans into complex glycans.

Fractionation of the subcellular organelles on isopycnic sucrose gradients showed that the human enzyme in the transformed cells banded at the same density—1.14 g/cm^{-3}, typical of Golgi cisternae—as the enzyme in the wild-type plant cells. These results demonstrate that complementation results from the presence of the human enzyme in the plant Golgi apparatus, where it is functionally integrated into the biosynthetic machinery of the plant cell. Thus, the mammalian enzyme is able to function in the plant context. It is not clear whether the enzyme is specifically targeted to the Golgi and retained there, or merely passes through the Golgi apparatus en route to a default destination. Given the evolutionary distance between plants and mammals and the great diversity of glycoproteins that are modified in each, there is probably no specific recognition between this Golgi enzyme and the polypeptide domains of the proteins it modifies. The biosynthesis of sialylated proteins will probably require the introduction of other genes besides the one for sialyltransferase: the cells need to synthesize sialic acid, convert it to CMP-sialic acid and transfer this nucleotide sugar across the Golgi cisternal membrane.

WHAT IS A PRACTICAL SHORT-RANGE STRATEGY?

We have described above some strategies for the production in plants of human glycoproteins with non-immunogenic or human-like glycans. Since many proteins of interest are secretory proteins, to which organelles should they be targeted and in which organs should they be expressed? Targeting to the vacuoles (protein bodies) in the seeds of dicotyledonous plants, or to the ER-derived protein bodies of cereals, would seem to give the highest chance of success. Seeds are conveniently harvested, stored and processed, and much work has been done on the use of seeds as heterologous expression systems. Seeds, and especially seed vacuoles, are very low in proteases compared to leaves and leaf vacuoles. Because pharmaceuticals have to be produced under highly controlled and reproducible conditions, it is likely that such plants would have to be grown in growth chambers rather than in greenhouses.

The removal of the GnT-I gene could be accomplished through the isolation of mutant plants from other species, e.g. *Brassica campestris* or *Pisum sativum*, in the same way as was done for *Arabidopsis*; alternatively, after the GnT-I

gene has been cloned from *Arabidopsis* and the homologue isolated from *Brassica napus*, sense or antisense repression of its expression could be attempted with this agronomic species.

Plant cell cultures grown in large volumes are also available for the production of recombinant proteins that could easily be purified after their secretion in the culture medium. We have recently shown that plant cells grown in the presence of a glycan processing inhibitor secrete glycoproteins without complex glycans. For instance, glycoproteins synthesized by plant cells in the presence of castanospermine have *N*-linked glycans exclusively of the high-mannose type. Protein biosynthesis and secretion and the viability of the cells were not affected by long-term (12 h) culture in the presence of the drug (Fitchette-Lainé et al., unpublished results). Thus plant cell systems are readily available for the production of recombinant glycoproteins with non-immunogenic glycans. An alternative approach is the *in vitro* 'humanization' of glycans made in plants. Indeed, plants without GnT-I activity, or plant cells treated with glycan processing inhibitors such as castanospermine, produce glycoproteins with high-mannose Endo H-sensitive glycans. Cleavage by Endo H completely removes a high-mannose glycan from a glycoprotein, except for the GlcNAc residue *N*-linked to the asparagine. This GlcNAc residue could be a basis for the *in vitro* synthesis of a mammalian-like glycan using the large panel of glycosyltransferases already available.

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